CHARACTERIZATION OF *STAPHYLOCOCCAL NUCLEASE* AND THE STATUS OF STUDIES ON ITS CHEMICAL SYNTHESIS

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As is true for so many fields of science (see for example the somewhat wistful article[1] by Gunther Stent on "the molecular biology that was") protein chemistry has recently undergone a large quantum jump. The dramatic progress in the methodology of sequence analysis and crystallography has introduced a new level of sophistication to the field and, except for very large protein molecules, particularly those containing many subunits, the direct approach of x-ray structure analysis combined with elucidation of covalent structure may soon bypass many of the more prosaic problems of protein chemistry. However, the question of the comparative structures of proteins in solution and in the crystalline state is not yet resolved, and the detailed study of the *dynamic* aspects of catalysis and conformation will undoubtedly require the more classical approaches for years to come.

An extracellular nuclease, produced by cultures of *Staphylococcus aureus*, has proved to be an extremely convenient and cooperative test object in our experimental approach to some of these questions. It has lent itself to a broad study of conformational stability and the mechanism of catalytic action, as well as crystallographic and organic synthetic investigations. Since I would like to spend a fair share of my time on some recent studies directed toward the chemical synthesis of large polypeptides, I shall give only a brief summary of our work on the characterization of the enzyme. Most of these findings were recently reviewed at the Brookhaven Symposium[2].

The protein, which can be conveniently isolated in large amounts from cultures, contains 149 amino acids and has a molecular weight of 16 807. Its complete sequence is shown in *Figure 1*. This globular enzyme contains no sulphhydryl groups or disulphide bonds and undergoes rapid and complete renaturation after treatment with denaturants or heat. A single tryptophan residue is buried deeply in the hydrophobic core of the molecule in the absence of denaturants, and two of the seven tyrosine residues appear to have a unique, cooperative function in the binding, and perhaps hydrolysis, of substrate molecules. Nuclease hydrolyses both DNA and RNA to produce 3′-mononucleotides and the principal attack is endonucleolytic with preferential cleavage of dXp–dTp and dXp–dAp bonds in DNA and Xp–Ap and Xp–Up bonds in RNA. The reaction catalysed is summarized in *Figure 2*, which also shows the structures of certain competitive inhibitors. Catalytic activity, as well as inhibitor binding, is completely dependent upon the presence of calcium ions (although Sr^{2+} will support the hydrolysis of DNA but not RNA).

Figure 1. The amino acid sequence of *Staphylococcal nuclease*

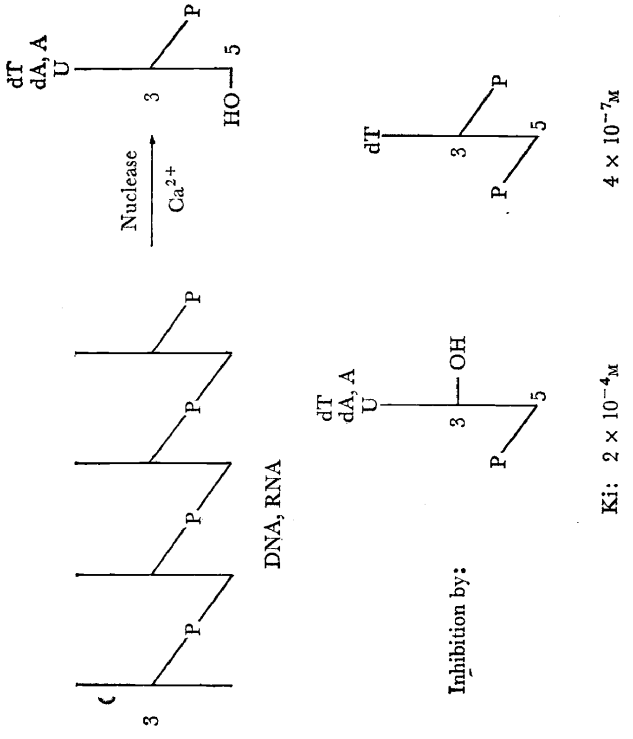

Figure 2. Reaction catalyzed by *Staphylococcal nuclease*

Kinetic studies on inhibition of DNA hydrolysis by a series of oligonucleotides of deoxythymidine bearing a phosphoryl group on the 5′-OH group of the terminal nucleotide have indicated that the substrate binding "groove" of the enzyme can accommodate a chain of approximately 3–4 monomers[3] (i.e., some 20–25 Å long) and the preliminary three dimensional structure at low resolution does indeed show such a region on its surface[4].

The most dramatic characteristics of the nuclease involve its capacity to bind ligands, and the consequences of such binding. The nucleotide ligands, the most effective of which is pdTp, bind in a 1:1 molar ratio in the presence of calcium ions. The dissociation constant of the nuclease–pdTp complex (about 4×10^{-7} M) obtained from the kinetics of inhibition of hydrolysis is the same whether DNA or RNA is used as substrate and this constant is very similar to that obtained by direct measurements of binding by gel filtration in the absence of substrate. Such results strongly indicate that the active site and the inhibitor binding site are the same. I should like now to summarize briefly some of the experimental consequences of this specific ligand attachment.

Crystal structure

As shown in *Table 1*, crystals of nuclease containing bound nucleotide and Ca ions are closely isomorphous with the native enzyme and both forms are convenient for heavy atom substitution.

Table 1. Unit cell dimensions of various crystal forms of *Staphylococcal nuclease*

Type I (no pdTp)	$a = b$ ↓	c ↓
Natural	47·8 ± 0·1 Å	× 63·55 ± 0·05 Å
$PtCl_6$	47·8 ± 0·1 Å	× 63·4 Å
$PtCl_4$	47·75 ± 0·1 Å	× 63·4 ± 0·1 Å
p-Cl—Hg—C_6H_4—SO_3H	47·7 ± 0·15 Å	× 63·45 ± 0·05 Å
p-$CH_3C(=O)$—O—Hg—C_6H_4—NH_2	47·7 ± 0·05 Å	× 63·45 ± 0·05 Å
Type II (1 mole/mole pdTp; 2 moles/mole Ca^{2+})		
Natural	48·2 ± 0·2 Å	× 63·25 ± 0·2 Å
Ba	48·25 Å	× 63·25 Å
Gd	48·0 Å	× 63·2 Å

Both Type I and Type II crystals are P_{41}; tetragonal with a 4-fold screw axis. These data were kindly made available by F. A. Cotton, A. Arnone, J. S. Richardson, D. C. Richardson and E. E. Hazen, Jr., MIT[4, 5].

Denaturation

The nuclease is strikingly protected against denaturation by urea and heat when nucleotide and calcium are present (*Table 2*) and alkaline denaturation occurs nearly one pH unit higher in the presence of the ligands.

Chemical reactivity

Native nuclease, after reaction with acetylimidazole[6], contains 5 *O*-acetyl tyrosine residues and 9–10 ε-*N*-acetyllysine residues. Almost all DNase and RNase activity is lost. Upon *O*-deacetylation, DNase activity is fully,

and RNase activity, partially restored. Only two tyrosyl and 8–9 lysyl residues are acetylated in the presence of pdTp and calcium ions and DNase activity is unimpaired. The small loss in RNase activity is restored upon *O*-deactylation.

Table 2. Protection against urea denaturation by nucleotide binding[2]

Rates of nuclease (0·041 mM) denaturation were measured by the fall in absorbancy at 237 mμ, except in the case of 2 M urea, where the increase at 244 mμ was used. "Immediate" effects occur in less than one minute.

Urea concentration	*Presence of*		*Rate constant* $sec^{-1} \times 10^2$
	T-3′,5′DiP 0·053 mM	Ca^{2+} 0·01 M	
2 *M*	—	—	5·9
	+	—	5·6
	—	+	6·0
	+	+	No denaturation
3 *M*	—	—	14·8
	+	+	No denaturation
4 *M*	—	—	Immediate
	+	—	Immediate
	—	+	Immediate
	+	+	4·6
6 *M*	—	—	Immediate
	+	+	10·5
8 *M*	—	—	Immediate
	+	+	29·4

Reaction in the absence of ligands with low levels (about 4 equivalents) of tetranitromethane[2] causes the specific *ortho* nitration of the tyrosine residue at position 85 and enzymic activity is abolished. In the presence of the ligands, tyrosine 85 is not nitrated and, instead, tyrosine 115 becomes unusually reactive to nitration in contrast to its complete lack of reactivity in the native protein. The mononitro-tyrosine 115 derivative has normal DNase activity, RNase activity is decreased by about 50 per cent, and tyrosine residue 85 has now entirely lost its *special* reactivity, even after removal of the ligands. A variety of spectrophotometric, fluorimetric, and solvent perturbation experiments have indicated a linked and reciprocal conformational change in the orientations of these two tyrosine residues during the addition of ligands, involving a shift from aqueous to hydrophobic environments for tyrosine 85, and the opposite for 115.

Spatial relationship of portions of the polypeptide chain

The specific mononitrotyrosyl derivatives may be isolated in chromatographically homogeneous form and converted to the corresponding amino-tyrosine derivatives by dithionite. The amino group thus produced has a pK of 4·7 and permits restricted crosslinking of the protein as summarized in *Figure 3.* Thus, a bifunctional reagent may be attached, at low pH, to the amino group without attack on other amino groups or hydroxyl groups

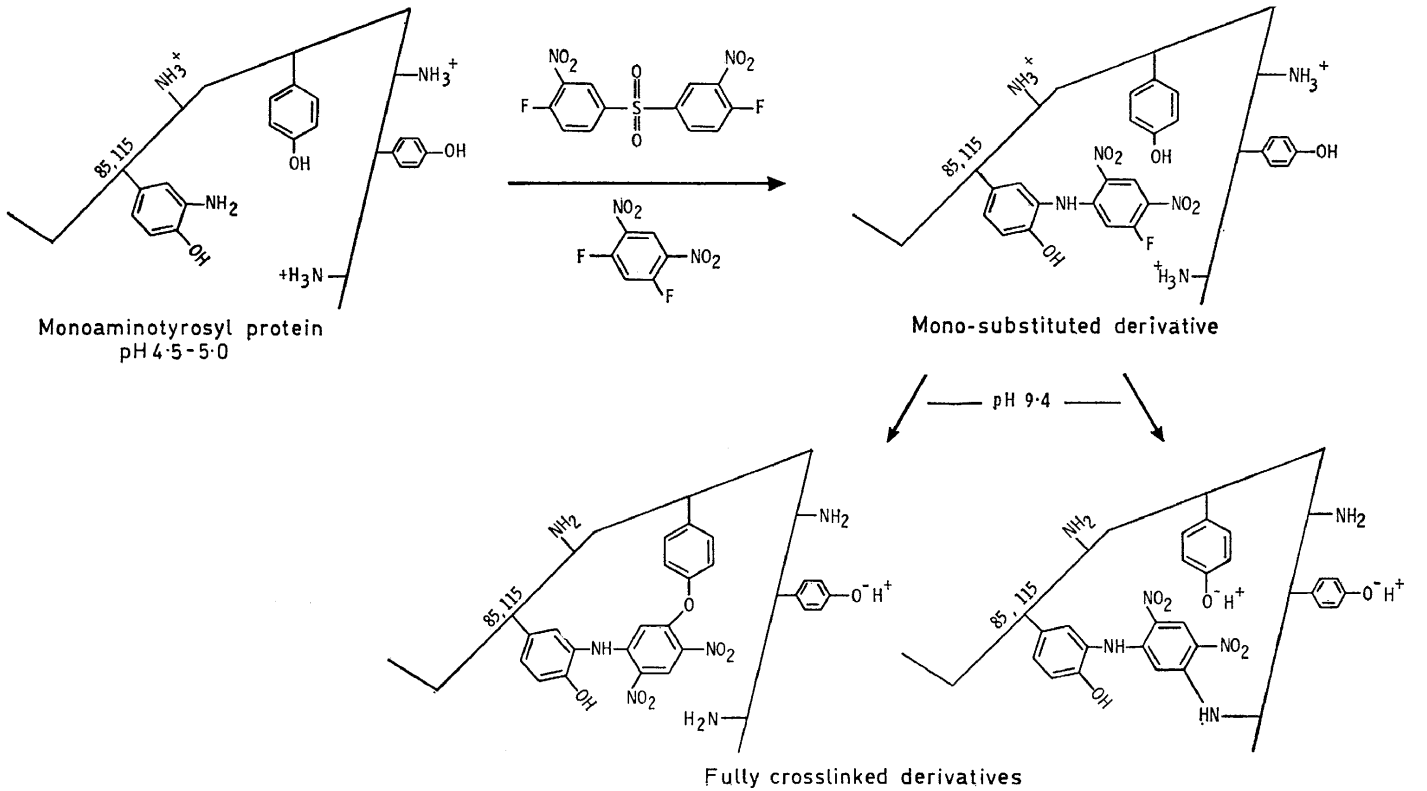

Figure 3. Specific crosslinking of the nuclease structure. As described in the text, a unique initial attachment of the reagents is possible because of the very low pK of the 3-NH_2 group of aminotyrosyl residues.

since these have much higher pK values. The pH may be then raised and the other end of the bifunctional reagent can now react with a stereochemically available unprotonated amino group, or with tyrosine, thus completing the cross-linking reaction. Such studies show that tyrosines 85 and 115 are indeed spatially adjacent, since tyrosine 85 cross links to tyrosine 115 with one of the reagents shown and to lysine 114 with the other[2].

Immunological properties

The binding of antibodies to nuclease, either as soluble or precipitating complexes, causes complete inactivation. The antigen–antibody reaction is strongly suppressed by ligand binding and soluble complexes can be partially dissociated by addition of pdTp and calcium ions. The exposed active site of the enzyme, therefore, appears to constitute a major antigenic site and slow precipitation in the presence of substrate analogues probably reflects reaction with the small amount of native protein in equilibrium with the liganded form[2, 7].

Susceptibility to proteolysis

Perhaps the most striking effect of the binding of inhibitors to the active site is the dramatic change in sensitivity to proteases. As shown in *Table 3*, the normally rapid digestion of the protein by a variety of proteases is nearly

Table 3. Effect of pdTp and Ca^{2+} on the susceptibility of nuclease to proteolytic digestion[2]

	Presence or absence of pdTp *and* Ca^{2+}	*Per cent of initial activity*		
		60 min	120 min	180 min
Subtilisin 10% w/w of nuclease	+	93	92	85
	−	11	5	5
α-chymotrypsin 2·5 w/w of nuclease	+	96	99	90
	−	35	11	8
Thermolysin 5% w/w of nuclease	+	77	73	72
	−	49	45	2

completely prevented by the addition of pdTp and calcium ions. The sites of attack of the protected protein by subtilisin, chymotrypsin and trypsin are shown in *Figure 4*. The products of the first two of these proteases can be isolated in pure form and have normal enzyme activity and unchanged physical properties. Indeed, preliminary studies on the crystal habit[4] of the subtilisin derivative suggest that this material may be isomorphous with the native crystals. The derivative produced with trypsin is of special interest in relation to the studies on organic synthesis which I shall describe below. In the presence of pdTp and calcium, trypsin cleaves the bond between residues

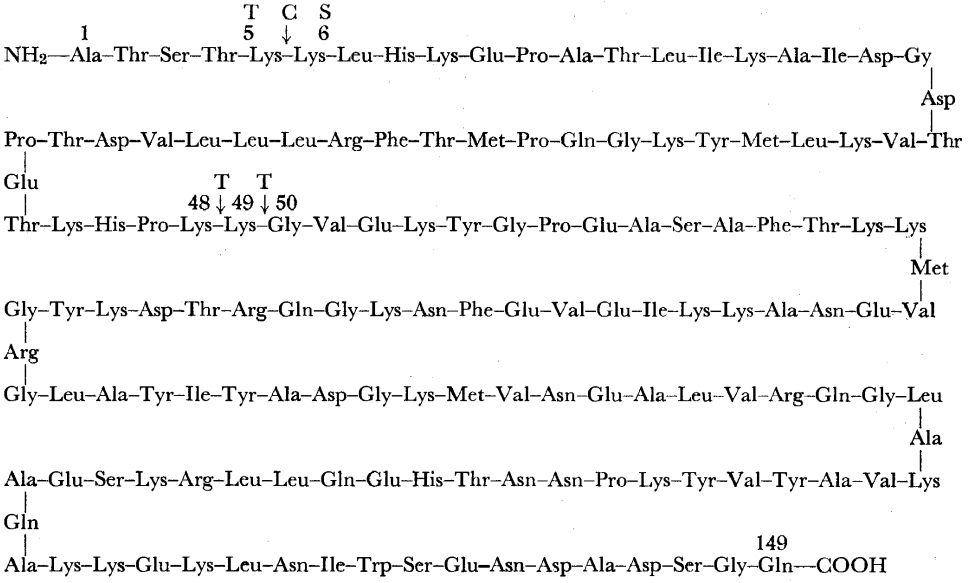

Figure 4. Sites of attack of trypsin (T), chymotrypsin (C) and subtilisin (S) on nuclease in the presence of pdTp and calcium ions

5 and 6 rapidly, yielding a derivative, Nase-T-p_{144}, which possesses normal enzyme activity. Subsequently, the bonds between residues 48–49 and 49–50 are cleaved, to yield two fragments, nuclease-T-p_2 (residues 6–48 or 6–49) and nuclease-T-p_3 (residues 49–149 or 50–149) (*Figure 5*). The two components interact through noncovalent bonds, with a dissociation constant[8] of approximately 10^{-7}. This complex, nuclease-T, exhibits approximately 8–10 per cent of the activity of the native enzyme. The two components may be separated by gel filtration in acid solution, and show the properties of unstructured random chains. They reassociate to regenerate full nuclease-T activity and the optical rotatory, immunologic and spectral properties of the parent complex. The reconstitution of active material is shown in *Figure 6*, and resembles closely the results of Richards and his colleagues with the two components of ribonuclease-S.

Nuclease-T has a much decreased stability to denaturing conditions. As shown in *Figure 7*, the effect of heat on the mean residue rotation at 233 mμ is significantly greater than on the native protein. Both curves are shifted to higher temperatures in the presence of pdTp and calcium ions[9].

The suppression of "motility"

K. Linderstrøm-Lang introduced the term motility in 1954 (see ref. 10) to describe a postulated flexibility in the three dimensional structures of proteins, suggested to account for the characteristics of hydrogen exchangeability in solution. Nearly all of the exchangeable hydrogens of nuclease exchange with solvent very quickly. However, if pdTp and calcium ions are added to an ^3H_2O solution of nuclease after complete equilibration with the tritiated solvent, a large number of hydrogen atoms become fixed in the structure and do not interact with the solvent[11]. This effect, much like the "corking" of a bottle, is quite consistent with the other ligand-induced effects mentioned above and supports the idea of an exposed groove in the surface of the protein which allows access to the core of the molecule. Indeed, the prevention of attack of nuclease in the presence of pdTp and Ca^{2+} by thermolysin (see *Table 3*), (an enzyme which has specificity only for bonds involving hydrophobic amino acids), also suggests a rather vulnerable "window" in the surface.

The organic synthesis of large polypeptide chains

It is quite possible that the application of the classical methods of peptide chemistry, involving the coupling of smaller, protected fragments, will ultimately result in the total synthesis of proteins. The synthesis of insulin, one of its chains containing 31 residues, thyrocalcitonin with 32, and ACTH with 39 have demonstrated the feasibility of this approach at the intermediate level. However, since even the smallest of the enzymes contain on the order of 100 residues, new techniques seem likely to be required to permit the kind of flexibility and speed needed in the future. Our own interest has been, for some time, the phenomenon of spontaneous folding of polypeptide chains into the unique, biologically active tertiary structures of the corresponding proteins[12, 13]. The systematic substitution of amino acid residues with others of different polarities and stereochemistry would clearly be of

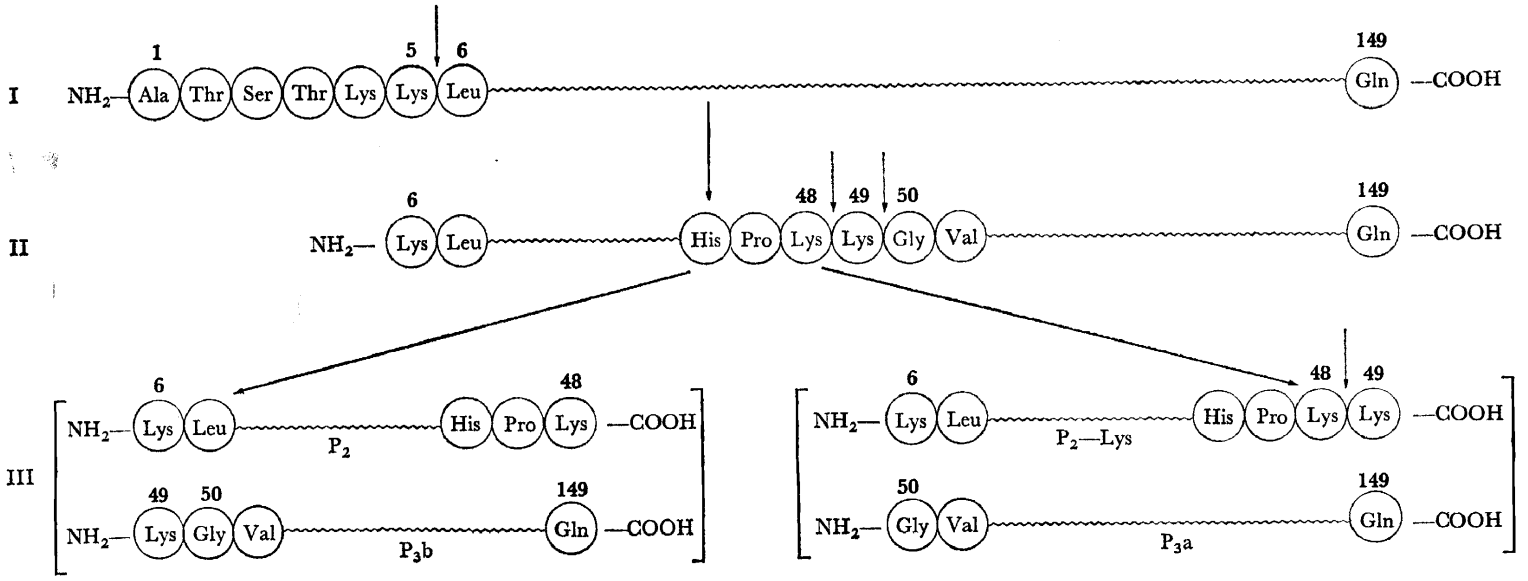

Figure 5. Steps in the formation of nuclease-T during trypsin digestion of ligand-protected nuclease

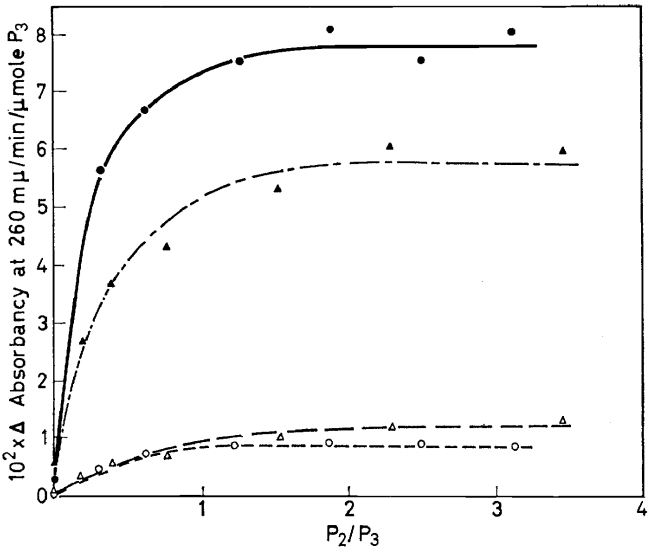

Figure 6. Reconstitution of enzymic activity during the addition of increasing amounts of fragment P_2 to a constant amount of fragment P_3 (see *Figure 5*). $P_2 + P_{3a}$: circles. $P_2 + P_{3b}$: triangles. The open points represent RNA hydrolysis and the solid points, DNA hydrolysis.

great value in the elucidation of the "geometric code" that relates sequence to conformation. Genetic mutation can give us some of these variants but a large, and particularly interesting class of replacements will undoubtedly be biologically lethal and not obtainable by such methods. We have therefore

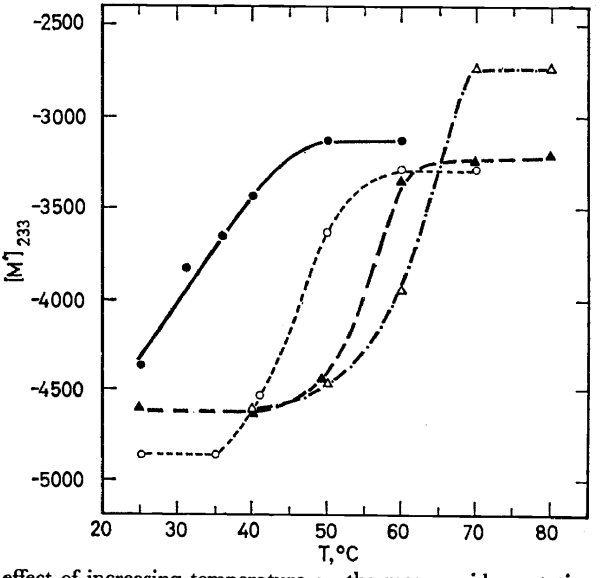

Figure 7. The effect of increasing temperature on the mean residue rotation of nuclease-T (●, without ligands; ○, in the presence of pdTp and calcium ions) and native nuclease (▲, without ligands; △, with ligands)

begun to examine methods that might permit the synthesis of the entire chain of staphylococcal nuclease, or at least useful fragments of its sequence. In the latter instance we have in mind the possibility of obtaining information on the nature of interactions that stabilize protein structure by examining, for example, the effect of selected portions of the sequence of nuclease on the binding affinity of fragments P_2 and P_3 of nuclease-T much in the way that haptens or fragments of antigens can inhibit antigen–antibody reactions. The "melting" of the structure of nuclease-T occurs over a low and fairly narrow temperature range (*Figure 7*), and shifts in such curves caused by analogues of even moderate sized peptide fragments might well furnish important thermodynamic data of the kind required in current efforts to compute geometry from sequence. Hofmann and his colleagues have, for example, recently shown[14] a striking effect on the binding of RNase-S-peptide to S-protein by a synthetic 13 residue fragment of S-peptide that contains 3-carboxymethyl histidine in place of the histidine residue normally present in position 12.

The solid phase technique[15]

Part of the somewhat negative reaction at a few recent meetings of peptide chemists to the solid phase method has been due to the fact that several well-recognized deficiencies exist in the method at the present time. Some of these—incompleteness of coupling at each monomer addition step, inadequacies of certain protecting groups, difficulties with the ultimate step of release from the resin support—are questions that I shall discuss in a moment. Unfortunately, much of the reaction has probably been due to the rather uncritical synthetic approach taken by many with a method in the process of evolution. The rapid, uncontrolled synthesis of a polypeptide of questionable homogeneity and low biological activity may justify announcement in the public press, but it does little to advance a field that requires precision and objectivity. I feel strongly that the solid phase principle will, when the difficulties of side reactions and coupling efficiency have been worked out, prove to be of enormous value in the solution of many problems in biology that require the complete synthesis of protein chains. A massive effort in many laboratories to improve the procedure is now in progress and the potentialities certainly justify the time spent.

The general procedure of solid phase peptide synthesis is outlined in *Figure 8*. The success of the method in the preparation of very long polypeptides is clearly dependent upon the completeness of reaction at each step of monomer addition and upon the stability of protecting groups to the reagents used during each cycle. Some evidence for a slight but significant loss of the ϵ-carbobenzoxy protecting group during HBr-TFA removal of t-Boc groups has been obtained by Yaron and Schlossman[16] and we have confirmed this mild sensitivity. Such a side reaction can lead to growth of branched chains and eventual problems in purification of the final product. We have examined, therefore, the use of the ϵ-trifluoroacetyl group for the protection of lysine residues[17, 18]. This group is apparently quite stable to the conditions employed and has been used extensively in the synthesis of a large number of protected fragments of the nuclease sequence[19]. The

stability of this NH_2-protecting group has also permitted us to carry out certain experiments on the estimation of coupling efficiency during successive monomer additions. For example, we have synthesized several oligolysines by the stepwise addition, using a threefold excess at each stage, of BOC-ε-TFA-lysine to a BOC-ε-TFA-lysyl-polymer. The final products were then released from the resin by treatment with HBr-TFA and subsequently treated with 1 M piperidine at 0° to remove TFA groups. The

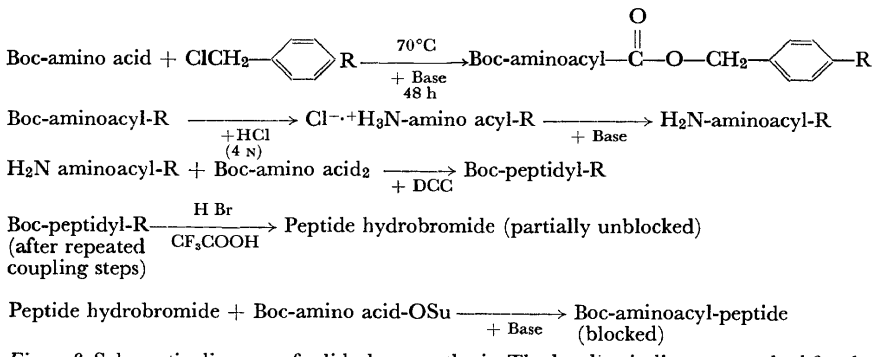

Figure 8. Schematic diagram of solid phase synthesis. The last line indicates a method for the conversion of solid phase products to protected peptides bearing a free terminal COOH group. (see also *Figure 11*)

total product was then subjected to chromatographic analysis on an ion exchanger[20] by an elution that can completely separate and quantitative oligolysines up to approximately 15 residues in length. "Heptalysine" was shown to contain 82 per cent of the heptamer, and 18 per cent of materials containing 6 or less residues of lysine. Similarly, "tetralysine" contained 94 per cent of the expected tetramer. These results are compatible with a coupling efficiency of approximately 98 per cent at each step. If these results are directly translatable to the case of longer peptides, a peptide of the length of nuclease fragment P_2, synthesized by the solid phase method, should contain $(0{\cdot}98^{42} \times 100)$ per cent of the desired sequence. The difficulty of purification of such a preparation, containing on the order of 40 or more closely related compounds differing by a single residue, is obviously not to be overcome by the usual physical procedures and special, highly selective methods will be required for isolation of the correct structure. One approach to the problem is considered below.

I should mention other uncertainties that arise in the appraisal of the purity of solid phase products. Most investigators using the method have observed that even rather long peptides yield good amino acid analyses and, when biological activity has been tested, that potency was frequently quite respectable. However it must be borne in mind that a mixture such as that mentioned above, differing only slightly in composition, would be expected to give excellent amino acid analyses since each residue may have been omitted with approximately the same frequency. Secondly, since high biological activity is often observed even after rather extensive modification of proteins and polypeptides, this criterion may be quite misleading. At the moment,

I would suggest that only the quantitative chromatographic analysis of specific enzyme digests—quantitative fingerprints in a sense—can give a true estimation of deviation from homogeneity.

Efforts are now underway in a number of laboratories to increase the coupling efficiency at each step, or to treat after each monomer addition in such a way as to terminate residual unlengthened chains. Another approach involves the development of other solid phase supports and the use of different kinds of activated monomers. We have for example, examined the addition of *N*-hydroxysuccinimide esters of protected monomers to several more hydrophilic polymeric materials such as multichain polyamino acids (*Figure 9*) or sepharose, with the purpose of removing the final products

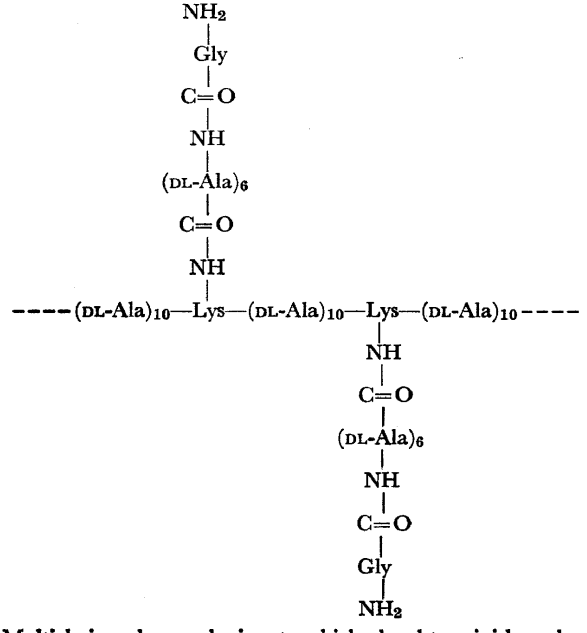

Figure 9. Multichain poly-D,L-alanine, to which glycyl termini have been attached.

by enzymic methods rather than by the usual HBr–TFA or liquid HF. An example of such a synthesis is shown in *Figure 10*, employing a relatively water soluble multichain polyalanine as support. Removal of the protected peptide was carried out using chymotrypsin and the final product, obtained in high yield, appeared to be homogeneous by several criteria. The approach is limited by the scarcity of completely specific proteases but the purity of the products obtained, together with the relative gentleness of the conditions, has led us to continue the exploration of this avenue.

Application of solid phase synthesis to nuclease

The successful application of the Merrifield procedure to the synthesis of a large biologically active polypeptide or protein must involve only such reagents for the final deprotection steps that are known not to inactivate the native material itself. *Table 4* summarizes the various blocking groups and

A. Synthesis

$$\text{Multichain DL-analine} \xrightarrow{\text{T-Boc-Gly-X}} \text{T-Boc-Gly-P} \xrightarrow[\text{alternately}]{\text{(a) Anhyd. TFA; (b) T-Boc-AA succ. ester}} \text{T-Boc} \text{ - - - - - - - } \text{X-Gly-P}$$

Condensations carried out in 66% ethanol; pH adjusted to 8·0 with Na_2CO_3; reaction performed at 5°C for 4–16 h using approximately 2·5-fold excess of N-OH-succinimide esters.

B. Enzymic cleavage (performed in pH-stat at pH 8).

(1) P–Gly–Leu - - - - - T–Boc $\xrightarrow[\text{lysin"}]{\text{"thermo}}$ P–Gly–Leu–NH_2 + T–Boc - - - - - COOH

(2) P–Gly–Tyr– / P–Gly–Phe - - - - - T–Boc $\xrightarrow[\text{trypsin}]{\text{Chymo}}$ P–Gly–NH_2 + { T–Boc - - - - - Tyr–COOH ; T–Boc - - - - - Phe–COOH

$$\text{T–Boc–Leu–Ala–Lys(}\epsilon\text{TFA)–Val–Ala–Tyr–Gly–Polymer}$$

A. Synthesis

μmoles/sample analyzed

	Lys	Leu	Val	Tyr
Step 1	0·21	—	—	0·24
Step 2	0·20	—	—	0·19
Step 3	0·18	—	0·17	0·16
Step 4	0·29	—	0·15	0·16
Step 6	0·46	0·21	0·22	0·22

B. Cleavage with chymotrypsin; base uptake corresponded to 91% of theory assuming pK = 8·0 for new NH_2-terminal Gly.

C. Analysis of LAP digest after deprotection with TFA and 1 M piperidine.

	Leu	Ala	Lys	Val	Tyr
μmoles	0·17	0·30	0·15	0·15	0·15

Figure 10. Proteolytic detachment of protected peptides from supporting polymers

Table 4. Blocking groups used in solid phase synthesis

Reactive group	*Blocking group*	*Conditions for removal*	*Stability of nuclease*
ε-amino (Lys)	TFA	Aqueous Piperidine	Stable
β,γ-carboxyl (Asp, Glu)	OBzl (ester)	HF, 0°C (also HBr)	Stable (inactivated by HBr)
Hydroxyl (Tyr, Ser, Thr)	Bzl (ether)	HF, 0°C (also HBr)	Stable
Imidazole (His)	im-Bzl	Na, liquid NH_3	Partial cleavage at Proline bonds
Guanidino (Arg)	NO_2	HF or H_2/Pd	Stable
α-amino (All residues)	Boc	Dilute HCl	Stable

reagents that we have employed in our studies. The convenient method of Sakakibara[21], employing liquid HF at temperatures between 0° and 20°, is fortunately permissible because of the resistance of the enzyme to incubation in this solvent. All the blocking groups that we have employed with the exception of the ε-TFA and the im-benzyl groups, may be efficiently removed with HF. The ε-TFA group is rapidly hydrolyzed by treatment with ice-cold aqueous piperidine[18]. Removal of the im-benzyl group on histidine residues can, unfortunately, only be accomplished with Na–liquid ammonia and this reagent, in our hands, has led to unacceptable levels of cleavage of bonds involving proline. Since nuclease contains 4 residues of methionine, catalytic hydrogenation is excluded. We have had some success with the im-carbobenzoxy group which is moderately stable to acid conditions and is readily removed by alkaline reagents (aqueous piperidine, for example). The use of the im-carbobenzoxy group is described below in connection with the synthesis of nuclease fragment P_2. Recent studies by Dr. David Ontjes suggest that im-dinitrophenyl may also possess suitable stability properties and its removal is easily accomplished by treatment with thiols.

Protection of the hydroxyl groups of threonine, serine and tyrosine by the *O*-benzyl function is generally employed although side reactions during coupling, with dicyclohexylcarbodiimide, of peptides with *unprotected* OH groups are minimal and may ultimately prove to cause a negligible degree of branching. Such difficulties, when they do occur, seem to be completely avoided when coupling is carried out using either *N*-hydroxysuccinimide esters (see for example *Figure 9*) or Woodward's reagent. Amino acid analyses of synthetic peptides containing tyrosine residues frequently give low values for this residue. Bromination of the ring during HBr–TFA cleavage may be minimized by passage of the HBr gas through resorcinol in TFA and, in the case of cleavage with HF, benzylation of aromatic residues in general is avoided by the inclusion of anisole as a benzyl cation trap.

Although there has, as yet, been insufficient experience with tryptophan-containing peptides, this residue appears to have quite adequate stability to both TFA and HF at room temperature for periods of two hours or more.

Synthetic strategy for the synthesis of nuclease-T fragment P_3

Several preparations of fragment P_2 of nuclease-T have been synthesized by the solid phase method and some results are summarized in a later section. The only mildly successful experience with this peptide, containing 42 residues, precluded the application of the unmodified procedure to fragment P_3, with 99 residues. The convenience of the solid phase method was therefore employed in the synthesis of short, protected fragments suitable for subsequent coupling. The principle of these syntheses is illustrated in the example shown in *Figure 11*. The stability of the ε-TFA blocking group to HBr–TFA permits the solid phase construction of fragments to which a protected, NH_2-terminal glutamic acid, aspartic acid, or other residue may be added after removal from the resin support. We have thus accumulated a series of protected peptides that comprise nearly all of the 99 residues of fragment P_3. The properties of one of these, representing residues 108–117

of the sequence, are shown in *Figure 12*. It should be emphasized that, to insure the purity of the final products, counter current distribution has generally been employed for the purification of peptides containing more than 5 or 6 residues.

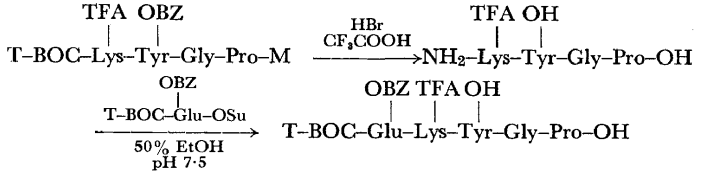

Figure 11. Solid phase synthesis with added termination step (M stands for "merrifield polymer").

Analysis of Peptide, Residues 108–117

	Amino acid analyses (molar ratios)									
	t-Boc-Leu-	Ala	TFA Lys	Val	Ala	Tyr	Val	Tyr	TFA Lys	Pro-OH
Acid hydrolysis of crude nonapeptide		1·12	1·04	0·98	1·12	0·94	0·98	0·94	1·04	1·00
Acid hydrolysis[a]	1·07	1·05	1·00	0·98	1·05	0·92	0·98	0·92	1·00	1·13
LAP after TFA, piperidine[a]	1·07	0·96	1·06	1·06	0·96	1·00	1·06	1·00	—	—

[a] Analyses of major countercurrent component. This component gave the following results on elemental analysis: *calc.* C, 54.99; H, 6.59; N, 11.65; F, 7.91; *found* C, 54.20; H, 6.23; N, 11.16; F, 7.81.

Figure 12. Analytical properties of a protected peptide fragment of nuclease prepared as described in *Figure 11*

Several stretches of the sequence, free of dicarboxylic amino acids with their susceptible benzyl ester protecting groups, have been made by removing the protected peptides from the solid phase support by hydrazinolysis (*Figure 13*)[22]. The products, after purification, are then immediately applicable to azide coupling. Hydrazinolysis is most convenient in those instances where the protected peptide hydrazide is rich in hydrophobic residues since the resulting nonpolar character permits ready removal of the large excess of hydrazine by washing with aqueous systems.

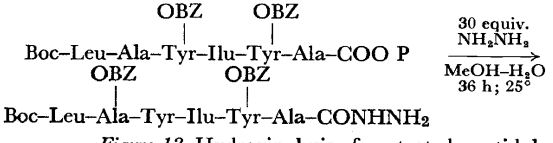

Figure 13. Hydrazinolysis of protected peptidyl-resin

The solid phase method, employed in the two modifications described above, has to date given no evidence of racemization, the absence of which has been determined by quantitative comparisons of acid and amino-peptidase digests.

The coupling problem

An ideal application of the solid phase method would appear to be the coupling of protected peptide fragments to the support, rather than amino acid monomers. By such a procedure, the cumulative effect of incompleteness of reaction at each step might be greatly minimized. *Figure 14* summarizes an example of such a coupling procedure. The properties of the product that was isolated indicated essentially quantitative coupling in this case, and racemization could not be detected. I understand that such a process has now actually been patented on the chance that it might prove to be really useful. Our own experiences to date have, at least, been quite encouraging.

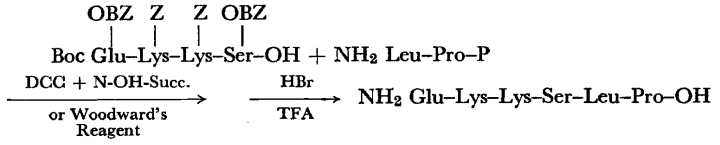

Figure 14. Fragment condensation by solid phase coupling

The safest and most direct path of synthesis at the moment, however, appears to be the use of the classical lengthening of the chain from the COOH-terminal end by successive addition of protected fragments such as those described above. *Figure 15* illustrates the stepwise synthesis of the fully

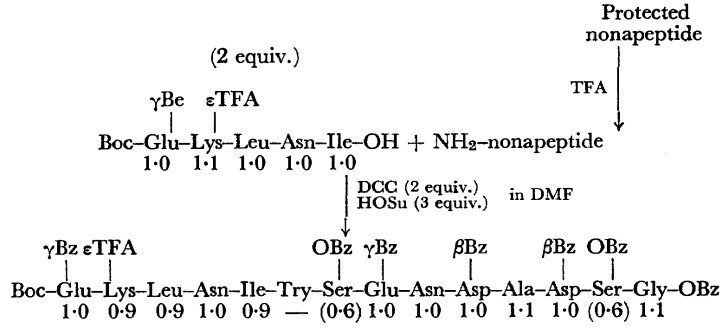

Figure 16. Coupling of a protected pentapeptide, prepared as indicated in *Figure 11*, with the COOH-terminal nonapeptide (*Figure 15*). Values for serine content are uncorrected for losses during acid hydrolysis

protected COOH-terminal nonapeptide fragment. The preceding five residues, synthesized by the solid phase method with terminal addition of protected glutamic acid, were then added as shown in *Figure 16*. As the growing chain is extended by the addition of further fragments it seems certain that solubility, even in favourable solvents such as dimethylformamide or dimethylsulphoxide, may become limiting. The growing chain may then become its own "solid phase". Earlier experiences that might help evaluate the chances of success in the total assembly of the 99 residue fragments are unfortunately lacking and one can only proceed with optimism.

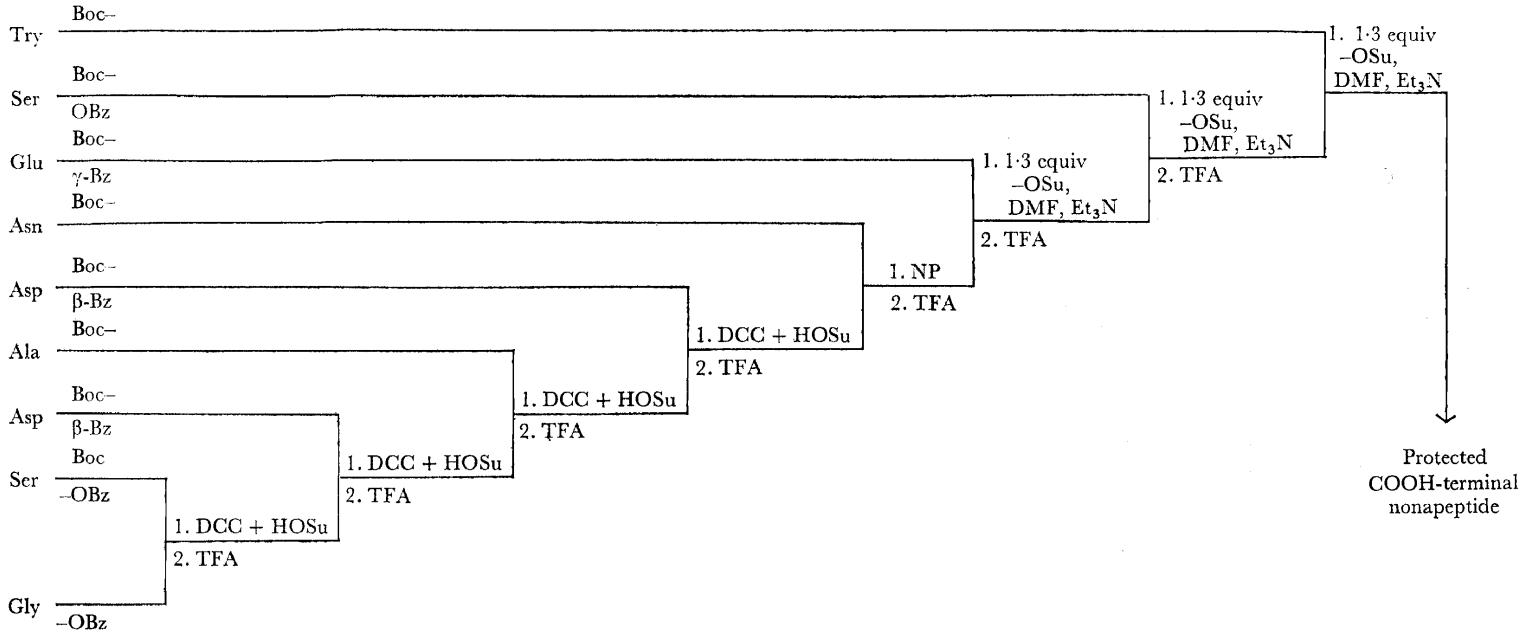

Figure 15. Classical synthesis of the protected COOH-terminal nonapeptide sequence of nuclease

"Functional" purification of synthetic peptides

Since the interactions that form and stabilize the structures of proteins are of very high specificity, an ideal approach to the unique isolation of a biologically active polypeptide might involve its capacity for complex formation. The opportunity to explore such methods for purification is, except for antigen–antibody systems, restricted at the moment to two examples; ribonuclease-S and nuclease-T. Both protein derivatives consist of two noncovalently bonded fragments which may be separated and recombined with full regeneration of the original activities of the complexes. Klaus Hofmann and his colleagues have effectively purified synthetic RNase-S-peptide by using this principle. In their experiments, S-peptide was recovered from the chromatographically purified RNase-S that resulted from the addition of synthetic S-peptide to natural RNase-S-protein.

We have recently prepared insolubilized RNase-S-protein and nuclease-T-P_3 (see *Figure 5*) by attachment of these enzyme fragments to CNBr-activated sepharose by the method of Porath[23]. The sepharose–polypeptide compounds form columns with excellent flow rates and with high capacity for binding the complimentary peptide fragments. These columns may be employed for the specific attachment of RNase–S–peptide or nuclease-T-P_2 from impure preparations and, after elution of the bound, purified materials, can be used repeatedly without loss in capacity. I should like to describe the properties of these materials and briefly to outline the results of some of our studies on RNase-S-peptide synthesized by the solid phase technique[24].

In a typical preparation, sepharose-4B (20 ml of bed volume) was washed thoroughly with water, suspended in water to a total volume of 40 ml, and the suspension adjusted to pH 11·0–11·5 with NaOH. A freshly prepared aqueous solution of CNBr (20 ml of a 25 mg/ml solution) was added and the pH maintained at approximately pH 11 for 4–5 minutes. After washing the suspension with water and 0·1 M $NaHCO_3$, coupling was carried out in 50 ml 0·1 M $NaHCO_3$ by addition of 4 ml 1 per cent S-protein solution in cold bicarbonate containing 0·01 M Na_2HPO_4. Complete coupling was achieved after 12–15 hours at 4°C.

The ability of columns prepared from such preparations to undergo successive stages of S-peptide binding and elution is illustrated in *Figure 17*. For these experiments, a column was prepared with 25 mg of the S-protein-sepharose complex and its activity was estimated by perfusion with a solution of 2′,3′-cytidine cyclic phosphate in 0·1 M Tris buffer, pH 7·5, employing a peristaltic pump set at a number of flow rates. The absorbancy increments at 284 mμ plotted against the reciprocal flow rates yield a straight line characteristic of the ribonuclease activity of the particular column. After initial measurements prior to addition of S-peptide, the column was perfused with 0·5 ml of a 0·6 per cent S-peptide solution and the activity was re-estimated. Finally, a third set of activity measurements was made after perfusion with 50 per cent acetic acid to remove specifically absorbed S-peptide. Although the original baseline was not reached, three successive binding-elution cycles yielded activities identical with those indicated by the upper two of the curves.

The capacity of the sepharose-S-protein columns was estimated, as shown

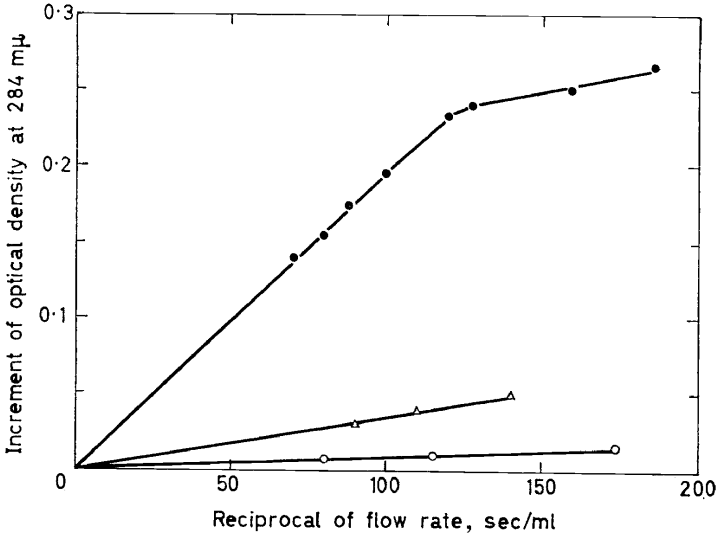

Figure 17. Activity measurements of ribonuclease-S-protein bound to a Sepharose column (see text for details)

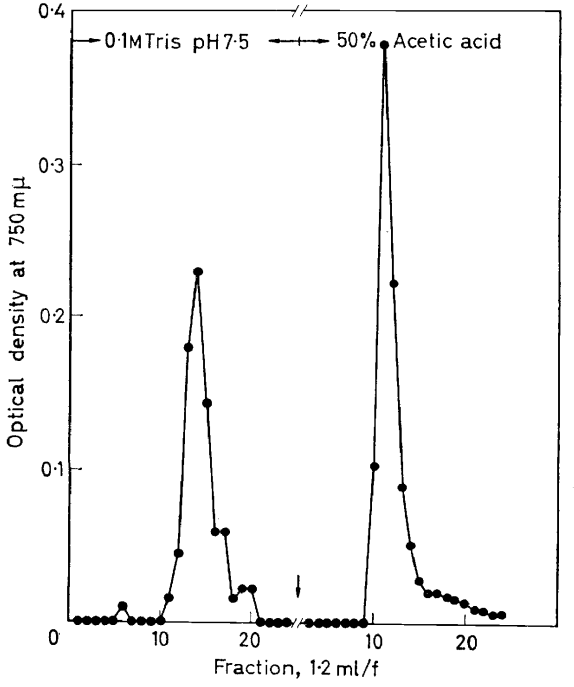

Figure 18. Estimation of capacity of RNase-S-protein column to bind S-peptide (see text for details)

in *Figure 18*, by passing an excess of S-peptide through a column containing 27 mg of bound S-protein. After thorough washing with buffer, 50 per cent acetic acid released 5 mg S-peptide. The bound S-protein was, thus, capable of tightly binding 45 per cent of its maximal capacity.

The use of such columns for the purification of synthetic S-peptide was then tested on two samples of the sequence of bovine pancreatic ribonuclease containing residues 1 through 15, as well as one analogue containing, as residue 16, a COOH-terminal alanine residue (*Figure 19*). The amino acid

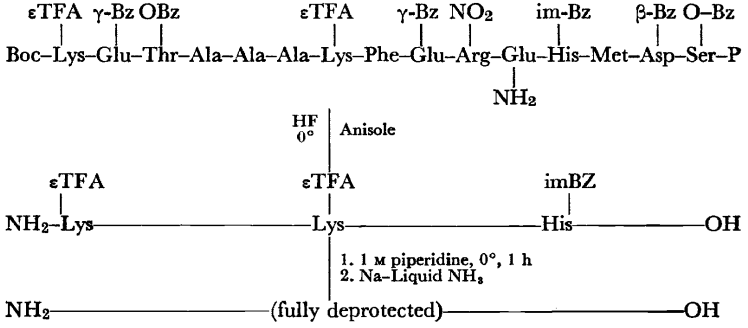

Figure 19. Solid phase synthesis of residues 1–15 of bovine pancreatic ribonuclease. It was shown earlier, both by classical synthesis (K. Hofmann and colleagues) and by carboxypeptidase digestion of natural S-peptide (residues 1–20) (J. Potts and C. Anfinsen), that the 15 residue compound possesses full biological activity

Table 5. Amino acid compositions of synthetic peptides[a] (residues 1–15 of bovine ribonuclease) prepared by solid phase procedures

Amino acid	*Prep.* II	*Prep.* III	*Theoretical*
Lys	1·76	1·74	2
His	0·45	1·02	1
Arg	0·32	0·69	1
Asp	1·04	1·05	1
Thr	0·73	0·72	1
Ser	0·70	0·70	1
Glu	2·63	2·57	3
Ala	3·00[b]	4·00[b]	3 (4[b])
Met	0·87	0·63	1
Phe	0·93	0·86	1

[a] After filtration through Sephadex G–25 to remove impurities of low molecular weight and marked hydrophobicity.
[b] Residues (1–15) grown on alanyl-polymer.

analyses of the fully deprotected preparations are given in *Table 5*. A number of amino acids deviate considerably from the theoretical values, trypsin digests yielded rather complex fingerprints only vaguely resembling maps of native digests, and the capacity to regenerate RNase activity when added to native S-protein was, in all cases low. When subjected to purification on insoluble S-protein columns, elution patterns such as shown in *Figure 20* were obtained. Typical amino acid analyses of the fractions eluted by 50

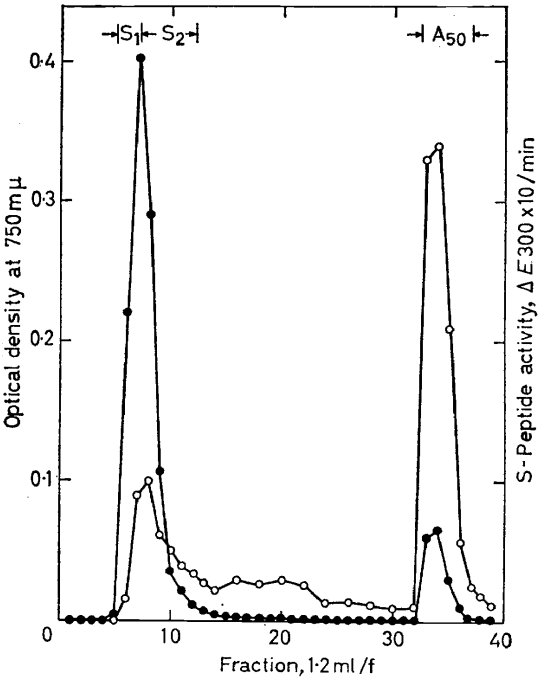

Figure 20. Purification of synthetic S-peptide derivative on a sepharose-S-protein column. After washing the column free of unbound material, tightly bound S-peptide was eluted with 50% acetic acid. Activity against 2′-3′-cytidine cyclic phosphate following addition of aliquots to a constant amount of S-protein, (○); polypeptide content of tractions by Lowry method, (●)

per cent acetic acid are summarized in *Table 6.* Whereas the original unfractionated product was incapable of producing full activation even at high ratios of synthetic peptide to S-protein, the fraction eluted with 50 per cent acetic acid gave 100 per cent of the theoretical activity at mole ratios on the

Table 6. Amino acid compositions of synthetic S-peptide derivatives before and after adsorption on Sepharose-S-protein columns, with subsequent elution by 50 per cent acetic acid.

	Preparation II (15 residues)		*Preparation* III (16 residues)	
	Before	*After*	*Before*	*After*
Lys	1·76	1·83	1·74	2·06
His	0·45	1·04	1·02	1·01
Arg	0·32	1·07	0·69	1·16
Asp	1·04	1·21	1·05	0·98
Thr[a]	0·73	1·10	0·72	1·24
Ser[a]	0·70	0·76	0·70	0·75
Glu	2·63	3·02	2·57	3·24
Ala[b]	3·00[b]	3·00[b]	4·00[b]	4·00[b]
Met	0·87	0·61	0·63	0·75
Phe	0·93	1·21	0·86	0·89

[a] Uncorrected for losses during acid hydrolysis.
[b] Based on Ala as 3·00 and 4·00 for preparations II, and III respectively.

order of 5:1 or less (*Figure 21*). Peptide maps of the A50 fraction were extremely similar to those produced with the native S-peptide except for the presence of two spots in the region corresponding to that tryptic fragment of the synthetic product having the sequence Gln–His–Met–Asp–Ser. These results, although preliminary, indicate the applicability of the purification method and the approach is being extended to synthetic samples of fragment P_2 of staphylococcal nuclease. Sepharose-bound fragment P_3 exhibits a high capacity for the reversible binding of fragment P_2.

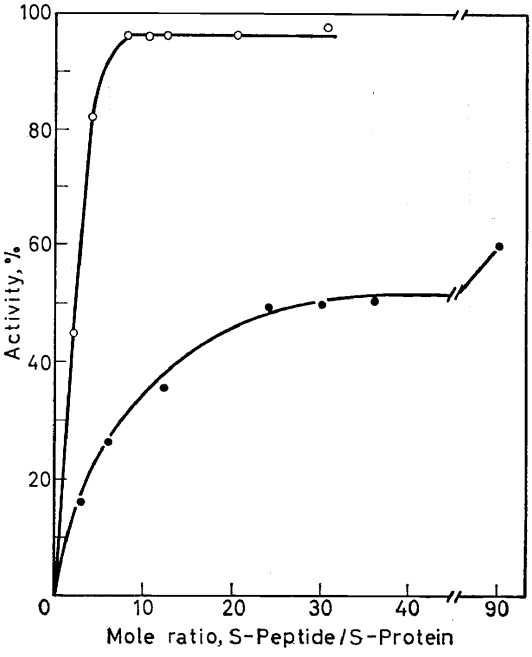

Figure 21. Activation of S-protein by crude (●) and purified (○) samples of synthetic S-peptide (residues 1–15).

Synthesis of fragment P_2 of nuclease-T

The COOH-terminal lysine residue of fragment P_2 may be removed by carboxypeptidase-B treatment to yield a fully active 42 residue polypeptide comprising residues 6–47 of the nuclease sequence[8]. The synthesis of this compound was attempted using several variations of the solid phase method. The blocking groups employed are indicated in *Figure 22*, together with a summary of the methods employed to remove the completed chain from the resin support, to complete deprotection, and to remove contaminating salts and other small molecules. Crude fragment P_2, which was obtained in a yield of over 75 per cent, still contained approximately 1 trifluoroacetyl group/molecule of peptide. Gel filtration through Sephadex G-25 or G-50 yielded two major fractions, the first to emerge from the column containing less than 0·5 equivalents of trifluoroacetyl- as judged from fluorine analyses. This fraction, when added to an aliquot of fragment P_3 which was itself devoid of activity, caused the formation of the active nuclease-T complex

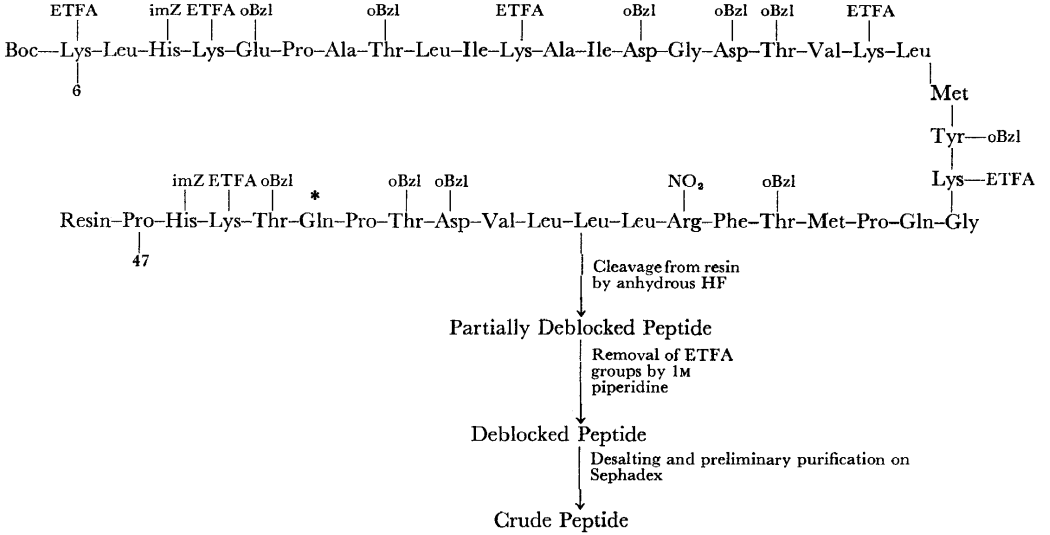

Figure 22. Solid phase synthesis of nuclease peptide P_2 residues 6–47

* We have recently discovered that this residue is actually glutamic acid, which may account for the low activity exhibited by the synthetic P_2 preparation.

with an efficiency that we judge to be on the order of 2–5 per cent of fragment P_2 isolated from native preparations of nuclease-T. The inherent activity of the preparation may be somewhat higher since we have obtained some evidence for the presence of inhibitory materials, possibly representing closely related isomers of the native peptide, which may compete for the P_3 fragment.

The synthetic peptide gave strong inhibition of the precipitin reaction between nuclease and its antiserum, a property that was completely destroyed by preliminary trypsin digestion. A peptide map of the trypsin digest showed, qualitatively, all the components obtained from digests of the peptide obtained from native nuclease-T. Polyacrylamide disc gel electrophoresis gave a single slightly widened band which migrated to the expected position.

Amino acid analyses of the two fractions from gel filtration are summarized in *Table 7* and, except for slightly low values for histidine and one or two other residues, were in good agreement with the expected values. Amino-terminal analysis by the "dansyl" method of Gray and Hartley showed only NH_2-terminal bis-dansyl-lysine.

Table 7. Amino acid analysis of deblocked nuclease P_2 peptide, residues 6 to 47

Amino acid	*Theory*	*Fraction A*	*Fraction B*
Lysine	6	6·4	7·5
Histidine	2	1·5	2·3
Arginine	1	1·1	0·8
Aspartic	3	3·4	3·2
Threonine	5	4·2	4·6
Glutamic + Glutamine	3	2·9	3·3
Proline	4	4·0	5·0
Glycine	2	2·2	2·0
Alanine	2	2·1	2·3
Valine	2	2·1	1·9
Methionine	2	1·7	1·5
Isoleucine	2	1·9	1·6
Leucine	6	6·1	6·8
Tyrosine	1	0·7	0·7
Phenylalanine	1	0·8	0·9
TFA's per mole by analysis for fluorine	none	0·5	1·3

It is clear that, at the present stage of development, the synthesis of a peptide of this length (see also the results on RNase-S-peptide described above) by the solid phase method is probably not capable of yielding material of adequate homogeneity for the study of the effects of side chain modification on biological activity or polypeptide interaction. We are at present repeating the synthesis with modifications employing, for example, the im-dinitrophenyl blocking group for histidine residues as well as coupling conditions that might increase the average efficiency of monomer addition at each step. Techniques described above for the "functional" purification of the synthetic fragment, together with careful application of ion exchange chromatography, may ultimately permit the isolation of suitably homogeneous material.

Acknowledgement

For completeness, I should mention that Dr. Meir Wilchek and Dr. Allen Zeiger are nearing the end of a laborious classical synthesis of the P_2 fragment and the comparison of the products made by the two methods should be of considerable interest.

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